

HAEMOLYSIS BY SERUM IN COMBINATION WITH CERTAIN BENZOL BODIES.

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While serum-complement acts as haemolysin in the presence of a specific immune body, and also along with colloidal silicic acid (Landsteiner and Jagic, Browning and Mackie), serum is also capable of producing lysis of red blood-corpuscles which have been treated with certain benzol bodies (Mackie (1), Browning and Mackie (2)).

In the original experiments it was found that if rabbit's corpuscles in suspension were treated with minute quantities of tetra-ethyl-diamino-triphenyl-methane sulphate (brilliant green) and then rabbit's serum in certain amounts was added, rapid haemolysis ensued. The amounts of brilliant green used were quite non-haemolytic by themselves. Table I shows the result of such an experiment. Even larger quantities of the dye are capable of producing only partial haemolysis by themselves, and amounts of serum which are insufficient to accelerate lysis with these quantities of brilliant green actually inhibit the haemolytic action of brilliant green by itself. An amount of brilliant green equal to ten times the smallest quantity necessary to produce lysis in the presence of serum produced only half-lysis of the test suspension, and that only after two hours' incubation at 37° C., while the same quantity of brilliant green in the presence of 0.1 c.c. of serum produced no effect. The lysis by brilliant green and serum was found to have attained its maximum in about a quarter of an hour even at room temperature.

Ox's corpuscles were found to be practically insusceptible to brilliant green by itself unless in large amounts, and in the presence of minute quantities of the dye rabbit's serum was capable of producing lysis of these corpuscles (Table II).

This effect, unlike haemolysis by serum + colloidal silicic acid, is not due to complement, since serum which has been heated at 55° C. for one hour, and serum from which complement has been removed by treatment with sensitised stomata or by cobra-venom (Omorokow's method), is as active as fresh serum (Tables III and IV).

As in the case of haemolysis by serum + colloidal silicic acid, "zone phenomena" are noticed (see Tables I, II, IV); thus there is an optimum

amount of brilliant green in the presence of which the haemolytic effect is produced with the minimum of serum.

Excess of the dye, therefore, may inhibit the action of the serum and necessitate larger amounts of serum being used to produce lysis. In fact, treatment of the serum by brilliant green before the addition of the corpuscles may abolish the hæmolytic action altogether, as shown in Table V. In carrying out these experiments, it was found that smaller amounts of serum were capable of producing haemolysis if the corpuscles were treated with the brilliant green for about a quarter of an hour before the addition of serum. Thus a certain length of time was required for the absorption of the dye by the corpuscles and the consequent physical or chemical alterations which rendered them susceptible. If the suspension which had been treated with brilliant green was centrifugalised, it was found that the bulk of the added dye was present in the supernatant fluid, and that when this was pipetted off and the corpuscular sediment washed with salt solution several times (till the supernatant fluid showed no tinting), and then re-suspended, the cells were still susceptible to the action of serum, and smaller amounts of serum were required to produce lysis than in the case of the original suspension. A treated suspension prepared in this way showed practically no green tinting. This suspension was found to be quite stable, and did not undergo spontaneous lysis; on the addition of serum, however, almost instantaneous lysis occurred.

Table VI shows the results of an experiment in which corpuscles were treated in this way: 1 c.c. of a 0.1 per cent. solution of brilliant green was added to 10 c.c. 5 per cent. suspension of washed ox's corpuscles (*i. e.* 0.05 c.c. to 0.5 c.c. suspension). The suspension was allowed to stand at room temperature for a quarter of an hour, and was then centrifugalised; the supernatant fluid was decanted and the sediment was washed several times with 0.85 per cent. NaCl till the washing fluid showed no tinting. The sediment after the last washing was suspended in 10 c.c. 0.85 per cent. NaCl., and showed practically no green coloration. The addition to 0.5 c.c. of this suspension of 0.025 c.c. of rabbit's serum produced immediate lysis. A parallel test was made with corpuscles to which brilliant green had been added (0.05 c.c. 0.1 per cent. solution to 0.5 c.c. suspension), as in the original experiments, and after the suspension had stood at room temperature for a quarter of an hour, varying amounts of serum were added to 0.5 c.c. of the suspension. Lysis only occurred in this case with larger amounts of serum.

As a further proof of the inhibiting effect of excess of brilliant green on the hæmolytic action of the serum, an experiment was carried out in which a fixed amount of heated rabbit's serum was mixed with varying quantities of brilliant green and then red blood-corpuscles treated with brilliant green were added. The result showed that serum in the presence of excess of brilliant green was incapable of exerting its haemolytic action (Table VII).

It has already been noted how the haemolytic action of the serum with brilliant green is not due to complement, since serum heated to 55° C. is still capable of acting. In order to ascertain whether this property was stable at higher temperature, tests were made with diluted rabbit's serum which has been boiled for two minutes. It was found that boiled serum was as active as fresh serum (Table VIII).

Rabbit's serum was also fractioned into (1) Albumin, (2) pseudo-globulin from "end-piece," and (3) "mid-piece" (by method of Browning and Mackie), and these fractions were tested separately and in combination with the treated corpuscles. As regards the haemolytic property of the serum, no qualitative fractioning occurred; all these protein fractions separately exerted a certain degree of haemolytic action which was less than that of the whole serum. When combined the full quantitative effect was reproduced, but only by "summation of effects" (Table IX).

Lecithin was also found to produce haemolysis of corpuscles treated with brilliant green (Table X).

That the haemolytic action of serum with brilliant green was not due to the action of lipoids (*e. g.* lecithin) present in it was proved by the fact that serum which had been extracted with alcohol and ether still retained its haemolytic activity (Table XI). Rabbit's serum was treated with a considerable excess of alcohol; the precipitated protein was removed by centrifugalisation and washed four times with a mixture of alcohol and ether; the precipitate was then suspended in a volume of salt solution representing five times the volume of the original serum.

Thus one must conclude that the active principle of the serum is an extremely stable one, and is resident in and distributed among the different proteins of the serum. It has been found possible to abolish the activity of the serum by filtering it through a Berkefeld filter. In this connection it is to be remembered that various enzymes are similarly retained in porcelain filters during filtration (Levy (3)), and, as Muir and Browning (4) originally showed, complement is removed from serum by the same process.

When the sera of a number of different animal species were tested with corpuscles of different species, it was found that the action of brilliant green in sensitising red corpuscles to the haemolytic effect of the serum was a general one, and there was no evidence of specificity as regards the action of any particular serum on the corpuscles of different species (Table XII). Thus brilliant green is capable of sensitising the corpuscles of an animal to the lytic action of its own serum, and the sera of the rabbit, guinea-pig, ox, sheep and man are all capable of lysing ox's corpuscles which have been treated with brilliant green to a more or less equal extent, though with some degree of variation. The lytic dose of sera of different species and of different individuals of the same species may vary from 0.005 c.c. to 0.05 c.c. The average dose of any serum may be said to be about 0.025 c.c. A

number of other substances have been tested with ox's corpuscles treated with brilliant green to ascertain whether other proteins, apart from those of serum, possess this property; varying amounts of egg albumen were added to ox's corpuscles which had been treated with different amounts of brilliant green. No haemolysis resulted. Peptone and gelatin solutions were also tested and found inactive. Other bodies which have been shown to play a part in various haemolytic phenomena were also tested. Thus varying amounts of brilliant green were added to ox's corpuscles which had been sensitised with 5 M.H.D. of a powerful haemolytic immune body (0.0025 to 1 c.c.) without effect. Colloidal silicic acid also showed no power of lysing corpuscles treated with brilliant green. The addition of varying amounts of brilliant green to venomised corpuscles also failed to produce lysis. Glucose solution had no haemolytic action on corpuscles treated with brilliant green.

Human cerebro-spinal fluid was found to be actively haemolytic to ox's corpuscles sensitised by brilliant green, though comparatively large amounts were necessary (0.1 c.c. to 0.25 c.c.); cerebro-spinal fluid was also found to have no inhibitory action on the lysis by the serum and brilliant green. Cerebro-spinal fluid heated for five minutes at 100° C. was as active as fresh cerebro-spinal fluid. This lysis occurred even when the fluid contained a minimal amount of protein, as evidenced by the absence of a precipitate or turbidity on the addition of an equal volume of 96 per cent. alcohol. These were all of course pathological cerebro-spinal fluids removed from cerebral and spinal cord cases for the Wassermann test. There was no correspondence between their activity with brilliant green and their power of reacting in the Wassermann test. Nos. 1 and 2 (Table XIII) contained small amounts of protein (shown by the alcohol test); No. 3 contained no appreciable amount of protein, and it was the most active, showing clearly that the effect does not depend on the presence of the serum proteins in the fluid. The fluid from a cystic swelling of the axilla was tested with corpuscles sensitised with brilliant green. This fluid was highly albuminous, and small quantities were sufficient to produce lysis (Table XIII). A richly albuminous urine was also tested. The albumin of 20 c.c. was precipitated by alcohol, removed by centrifugalisation, and suspended in 3 c.c. 0.85 per cent. NaCl. It was found that 0.5 c.c. of this suspension was capable of producing lysis of ox's corpuscles treated with brilliant green (Table XIV).

The susceptibility of ox's red blood-corpuscles to laking by hypotonic salt solution, acid and alkalies was investigated. Thus it has been shown that in certain pathological states, alterations occur as regards the susceptibility of human erythrocytes to various laking agents (McNeil and others) (5). It was found, however, that ox's corpuscles treated with brilliant green exhibited the same degree of susceptibility to hypotonic salt solution as normal corpuscles.

A very peculiar change was, however, noted as regards the susceptibility of the treated corpuscles to the lytic action of acid. Alkali was not, however, found to be more haemolytic than in the case of normal ox's corpuscles. Varying amounts of $\frac{N}{100}$ HCl and $\frac{N}{100}$ NaOH were added to 1 c.c. ox's corpuscles sensitised with brilliant green, and in parallel series the same amounts were tested with 1 c.c. suspension of normal ox's corpuscles. In one experiment $0.075 \frac{N}{100}$ HCl produced lysis of the treated corpuscles, while 0.25 c.c. was required to haemolyse 1 c.c. of the untreated suspension. Thus the treated corpuscles are more than three times as susceptible to the action of acid. There is, however, no difference in susceptibility to the action of $\frac{N}{100}$ alkali.

While these experiments have been carried out with brilliant green, other allied benzol compounds have been found to behave in a similar fashion, *e.g.* diamino-triphenyl-methane-hydrochloride (Döbner's violet), tetra-methyl-diamino-triphenyl-methane (malachite green), hexa-ethyl-tri-amino-triphenyl-methane (ethyl violet), and hexa-methyl-triamino-triphenyl-methane (methyl violet).

It has thus been shown how serum possesses certain haemolytic properties apart from the action of the labile complement; as in the case of haemolysis by complement the serum, of course, is incapable of affecting the intact corpuscles, and just as the sensitising effect of immune body or colloidal silicic acid renders the red cells susceptible to the toxic action of the complement, so also these benzol bodies bring about some sensitisation of the corpuscles. It is impossible to say what this sensitisation depends on. It is certain that physical alterations of the stromata are produced and that these bodies have the power of slowly damaging the corpuscles, but the experiments clearly demonstrate that there is a thermo-stable property or constituent of serum concerned in affecting lysis of red blood-corpuscles, provided some preliminary physical alteration is produced. The ultimate result is not of the nature of a disintegration of the cells, since the stromata are still visible under the microscope after haemolysis, as in the case of complement-immune-body haemolysis. It is also remarkable that minute amounts of serum (*e.g.* 0.005–0.025 c.c.) are capable of lysing the treated corpuscles. While lecithin possesses similar qualities, it cannot be said that the action of the serum depends on its lipoids. Moreover, though, in the case of serum, this peculiar property is indissociable from its proteins, and may be resident in the proteins excreted in a pathological urine, it is doubtful whether it is directly attributable to them, since cerebro-spinal fluids which were practically devoid of proteins are capable of lysing the sensitised corpuscles even in comparatively small amounts.

TABLE I.

Lysis of 1 c.c. 5 per cent. Suspension Washed Rabbit's Corpuscles.

		Rabbit serum (same animal).			
		0·1 c.c.	0·2 c.c.	0·4 c.c.	No serum.
Brilliant green 1 per cent. aqueous solution rendered isotonic immediately before use by addition of appropriate amount of 10 per cent. NaCl.	c.c. 0·01	0	Trace	Distinct	0
	0·025	0	Just complete	Complete	0
	0·05	0	0	Complete	0

Readings after ¼ hour at 37° C.

TABLE II.

Lysis of 0·5 c.c. 5 per cent. Suspension Washed Ox's Corpuscles.

		Rabbit serum.				
		0·025 c.c.	0·05 c.c.	0·1 c.c.	0·2 c.c.	No serum.
Brilliant green 0·1 per cent. solution (isotonic)	c.c. 0·075	Distinct	Complete	Complete	Complete	0
	0·1	"	"	"	"	0
	0·125	0	Marked	"	"	0

Control: 0·5 c.c. ox's red blood-corpuscles + 0·5 c.c. rabbit serum = no lysis.
Readings after ¼ hour at 37° C.

TABLE III.
Lysis of 0.5 c.c. 5 per cent. Suspension of Washed Rabbit's Corpuscles + 0.075 0.1 per cent. Solution of Brilliant Green (Isotonic).

	Rabbit's serum.				Controls.
	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	No serum.
Unheated serum	Very marked	Almost complete	Complete	Complete	0
Serum heated at 55° C. for 1 hour	Ditto	Complete	"	Distinct	0
Serum treated with ox's blood-corpuscle stromata + 10 doses of immune body (1½ hours at 37° C.)	"	"	"	"	0
					0.2 c.c. serum + 0.5 c.c. 5 per cent. ox's blood + 5 doses of immune body = complete lysis.
					0.6 c.c. serum + 0.5 c.c. 5 per cent. ox's blood + 5 doses of immune body = no lysis.
					0.6 c.c. serum + 0.5 c.c. 5 per cent. ox's blood + 5 doses of immune body = no lysis.

TABLE IV.
Lysis of 0.5 c.c. 5 per cent. Suspension Washed Ox's Corpuscles.

	Fresh rabbit's serum.				Serum treated with cobra venom.			No serum.
	0.025 c.c.	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.025 c.c.	0.05 c.c.	0.1 c.c.	0.2 c.c.
Brilliant green 0.1 per cent. solution (isotonic) { 0.075 c.c. 0.1 c.c. 0.125 c.c.	0	Almost complete	Complete	Complete	Distinct	Complete	Complete	Complete
	Very marked	Complete	"	"	"	Almost complete	"	"
	Trace	Marked	"	"	0	Marked	Almost complete	"
								0
								0
								0

Controls: 0.5 c.c. ox's corpuscles + 0.5 c.c. rabbit's serum = no lysis.
 " + 5 IB + 0.2 rabbit's serum = complete lysis.
 " + 0.2 c.c. rabbit's serum treated with venom = no lysis.

TABLE V.

A.—0·5 c.c. 5 per cent. Suspension Rabbit's Corpuscles + 0·1 c.c. of 0·1 per cent. Solution Brilliant Green incubated together for $\frac{1}{4}$ hour at 37° C.

+ Rabbit's serum.			
0·05 c.c.	0·1 c.c.	0·2 c.c.	0·3 c.c.
Complete lysis	Complete lysis	Complete lysis	Complete lysis.

B.—0·1 c.c. of 0·1 per cent. Solution of Brilliant Green + Varying Amounts of Rabbit's Serum incubated together for $\frac{1}{4}$ hour at 37° C. and then 0·5 c.c. 5 per cent. Suspension of Rabbit's Corpuscles added.

Rabbit's serum.			
0·05 c.c.	0·1 c.c.	0·2 c.c.	0·3 c.c.
No lysis	No lysis	No lysis	No lysis.

TABLE VI.

Lysis of 0·5 c.c. 5 per cent. Suspension Ox's Corpuscles.

	Heated rabbit's serum (55° C.).				
	0·25 c.c.	0·05 c.c.	0·1 c.c.	0·2 c.c.	No serum.
Brilliant green 0·05 c.c. 0·1 per cent. solution	0	Marked	Complete	Complete	0

Control : 0·5 c.c. ox's corpuscles + 0·5 c.c. heated rabbit serum = no lysis.

Lysis of 0·5 c.c. 5 per cent. Suspension Ox's Corpuscles treated with Brilliant Green (0·05 0·1 per cent. Solution per 0·5 c.c. Suspension) and washed free of Excess by Method detailed in Text.

+ Heated rabbit's serum (55° C.).				
0·025 c.c.	0·05 c.c.	0·1 c.c.	0·2 c.c.	No serum.
Just complete	Complete	Complete	Complete	0

TABLE VII.

Lysis of 0.5 c.c. 5 per cent. Suspension of Ox's Corpuscles treated with Brilliant Green (0.05 0.1 per cent. Solution per 0.5 c.c. Suspension and washed free of Excess).

+ Heated rabbit's serum (55° C.).				
0.005 c.c.	0.1 c.c.	0.025 c.c.	0.05 c.c.	No serum.
Almost complete	Complete	Complete	Complete	0

+ Varying amounts of brilliant green 0.1 per cent. solution (isotonic).

	0.05 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.
0.025 c.c. rabbit's serum (heated)	Almost complete	0	0	0
No serum	0	0	0	0

Control : 0.5 c.c. ox's corpuscles + 0.5 c.c. heated rabbit's serum = no lysis. (*Vide* text for explanation.)

TABLE VIII.

Lysis of 0.5 c.c. 5 per cent. Suspension Ox's Corpuscles treated with Brilliant Green.

	0.025 c.c.	0.05 c.c.	0.1 c.c.	No serum.
Fresh rabbit's serum .	Almost complete	Complete	Complete	0

	0.125 c c	0.25 c.c.	0.5 c.c.	
Boiled serum, 1 : 5 .	Just complete	Complete	Complete	0

Control : 0.5 c.c. ox's corpuscles + 0.5 c.c. rabbit's serum = no lysis.

TABLE IX.

Lysis of 0.5 c.c. 5 per cent. Suspension of Ox's Corpuscles treated with Brilliant Green.

	0.025	0.05 c.c.	0.1 c.c.	No serum.
Rabbit's serum	Just complete	Complete	Complete	0
Albumin	Distinct	Very marked	"	0
Globulin from "end piece"	0	Marked	"	0
"Mid piece"	Marked	Just complete	"	0
Three fractions combined	Just complete	Complete	"	0

Control: 0.5 c.c. ox's corpuscles + 0.5 c.c. rabbit's serum = no lysis.

TABLE X.

Lysis of 0.5 c.c. 5 per cent. Suspension Ox's Corpuscles treated with Brilliant Green.

	0.025 c.c.	0.05 c.c.	0.075 c.c.	0.1 c.c.	0.15 c.c.	No lecithin.
Lecithin 0.75 per cent. alcoholic solution 1 part in 8 parts of 0.85 per cent. NaCl	0	Trace	Marked	Very marked	Complete	0
<i>Control: Alcohol 1 part in 8 parts 0.85 per cent. NaCl</i>	0	0	0	0	0	0

Control: 0.5 c.c. ox's corpuscles + 0.3 c.c. lecithin 1 : 8 = no lysis.

TABLE XI.

Lysis of 0.5 c.c. 5 per cent. Suspension Ox's Corpuscles treated with Brilliant Green.

	0.025 c.c.	0.05 c.c.	0.1 c.c.	No serum.
Fresh rabbit's serum	Almost complete	Complete	Complete	0
	0.125 c.c.	0.25 c.c.	0.5 c.c.	
Serum 1.5 extracted with alcohol and ether	Marked	Almost complete	Complete	

TABLE XII.
Lysis of 0.5 c.c. Suspension Ox's Corpuscles Treated with Brilliant Green.

	0.025 c.c.	0.05 c.c.	0.1 c.c.	0.2 c.c.	No serum.	Lysis of 0.5 c.c. ox's corpuscles.
Rabbit's serum (a)					—	0
Guinea-pig's serum	Almost complete	Complete	Complete	Complete	0	0
Ox's serum	Complete	"	"	"	0	0
Sheep's serum	"	"	"	"	0	0
Human serum (1)	Marked	Almost complete	"	"	0	0
" (2)	Trace	Complete	0	0	0	0
" (3)	Marked	"	0	0	0	0
" (4)	Distinct	"	0	0	0	0
"	Complete	0	0	0	0	0

Lysis of 0.5 c.c. Suspension of Sheep's Corpuscles Treated with Brilliant Green.

	0.005 c.c.	0.01 c.c.	0.025 c.c.	0.05 c.c.	No serum.
Rabbit's serum (a)					
Human serum (4)	0	Marked	Complete	Complete	0
	Complete	Complete	0	0	0

Lysis of 0.5 c.c. 5 per cent. Suspension of Guinea-pig's Corpuscles Treated with Brilliant Green.

	0.005 c.c.	0.01 c.c.	0.025 c.c.	0.05 c.c.	No serum.
Human serum (5)	Trace	Marked	Complete	Complete	0

TABLE XIII.
Lysis of 0.5 c.c. Suspension Ox's Corpuscles treated with Brilliant Green.

Human.	0.025 c.c.	0.05 c.c.	0.075 c.c.	0.1 c.c.	0.15 c.c.	0.2 c.c.	No C.S.F.	0.5 c.c. suspension of ox's cor- puscles: no brilliant green.	
								0.2 c.c.	
Cerebro-spinal fluid (1)	0	Distinct	Marked	Very marked	Almost complete	Complete	0	0	
Cerebro-spinal fluid (2)	0	Trace	Distinct	Marked	Very marked	Complete	0	0	
Cerebro-spinal fluid 100° C.	0	Distinct	Marked	Very marked	Complete	Complete	0	0	
Cerebro-spinal fluid (3)	Marked	Almost complete	Complete	Complete	0	0	0	0	
Cerebro-spinal fluid (4)	Marked	Very marked	Very marked	Almost complete	Complete	0	0	0	
Cystic fluid . . .	0.065 Trace	0.075 Marked	0.01 Complete	0.05 Complete	—	—	—	0.5 = 0	

TABLE XIV.

Lysis of 0.5 c.c. 5 per cent. Suspension Ox's Corpuscles treated with Brilliant Green.

	0.025 c.c.	0.075 c.c.	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.5 c.c.	0.
Albumin from urine suspended in 0.85 per cent. NaCl. (<i>Vide</i> text)	0	0	0	Trace	Dis- tinct	Com- plete	0

Control: 0.5 c.c. ox's corpuscles + 0.7 c.c. suspension albumin from urine =
no lysis.

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